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EFFECTS OF LOCAL ANESTHESIA ON GINGIVAL CAMP LEVELS.(U)
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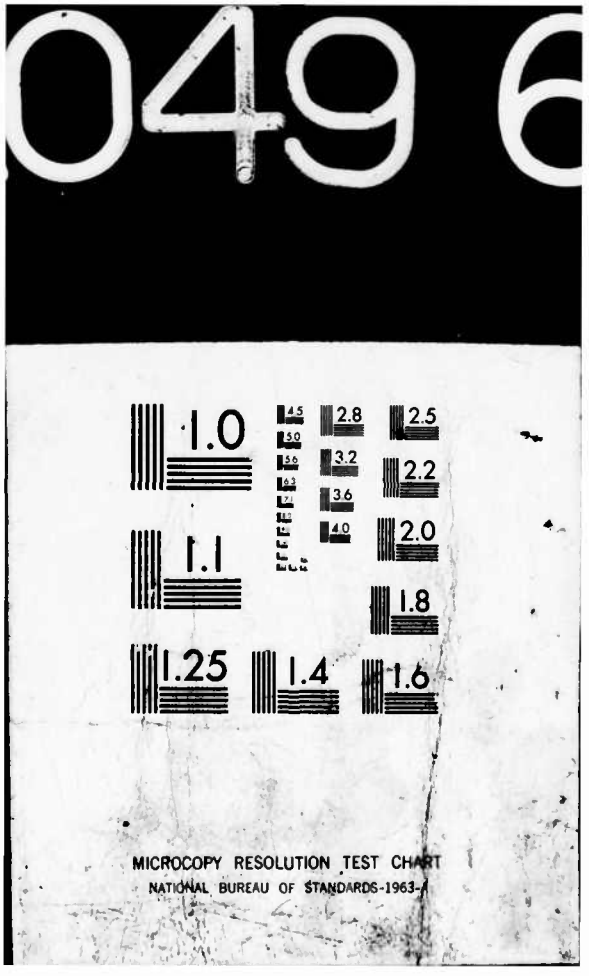
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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER (6)

2. GOVT ACCESSION NO. (12) B.S.

3. RECIPIENT'S CATALOG NUMBER (9)

4. TITLE (and Subtitle)
EFFECTS OF LOCAL ANESTHESIA ON GINGIVAL cAMP LEVELS.

5. TYPE OF REPORT & PERIOD COVERED
Manuscript (Rept. for)
1 JAN 76 - 2 FEB 78,

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s) (10)
Marvin F. Grower, Dorothy Chandler, George Kramer, and John A. Stow

8. CONTRACT OR GRANT NUMBER(s)

9. PERFORMING ORGANIZATION NAME AND ADDRESS
U. S. Army Institute of Dental Research
Walter Reed Army Medical Center
Washington, DC 20012

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
61102A, 3A161102B71R, 04, 086 (16) (17)

11. CONTROLLING OFFICE NAME AND ADDRESS
U. S. Army Medical Research & Development Command
HQDA (SGRD-RP)
Washington, DC 20314

12. REPORT DATE (11)
2 FEB 78

13. NUMBER OF PAGES
22

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)
(12) 25 p.

15. SECURITY CLASS. (of this report)
UNCLASSIFIED

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)
This document has been approved for public release and sale; its distribution is unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES
None

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)
cAMP, monkey, gingiva, plasma, Lidocaine, local anesthesia, epinephrine.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
The base levels of cAMP in the attached gingiva of Rhesus monkeys and the changes in tissue cAMP levels produced by infiltration anesthesia with Lidocaine and Lidocaine containing 1:100,000 epinephrine was studied. The basal level of cAMP in uninjected monkey gingiva ranged from 12-20 picomoles cAMP/mg of gingival protein. This level was 75 times greater than the cAMP content of monkey blood plasma. Infiltration of the attached gingiva with saline or plain Lidocaine for 5 min did not produce any significant changes in tissue cAMP levels. Infiltration of the gingiva with Lidocaine containing 1:100,000 epinephrine, on the

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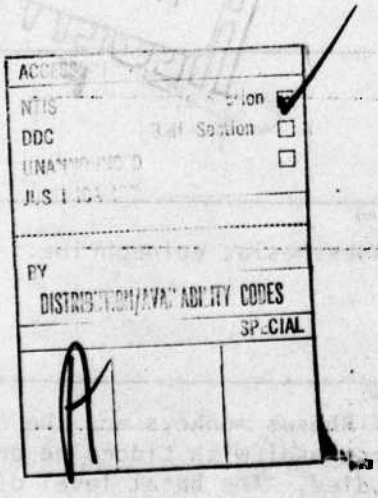
other hand, caused a very marked increase in tissue cAMP levels. Thirty seconds after infiltration with Lidocaine with 1:100,000 epinephrine there was a 150% increase in cAMP content of the anesthetized tissue versus the uninjected control tissues. The maximal increase in tissue cAMP levels was observed 5 min. after infiltration when the cAMP content of the gingiva was 1000-1100% above the control level. It is proposed that regulation of tissue cAMP levels by epinephrine or other agents may prove of therapeutic usefulness in regulating inflammation and healing of tissues after surgery or other trauma.



EFFECTS OF LOCAL ANESTHESIA ON GINGIVAL cAMP LEVELS

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EFFECTS OF LOCAL ANESTHESIA ON GINGIVAL cAMP LEVELS

INTRODUCTION

Gingival surgery and other dental procedures frequently require the infiltration of local anesthetics into the surrounding tissues. The inclusion of epinephrine in the anesthetic has been shown to prolong the duration and effect of the anesthesia and to reduce the bleeding from the tissues due to its vasoconstrictive effect.

Recently, Yoshikawa, *et al.*,[1] showed that *in vitro* incubation of pig or human epidermis with epinephrine increased epidermal cAMP levels. cAMP metabolism is of great importance in every mammalian tissue[2] and because of this and the frequent use of epinephrine containing anesthetics in dentistry, we decided to see what effect infiltration of the gingiva with local anesthetic and anesthetic containing epinephrine had on tissue cAMP levels.

Data relevant to these possible effects was needed to ascertain if local anesthesia infiltration could be used therapeutically to regulate gingival cAMP levels during periodontal or other surgical procedures. Also, such information was needed to further standardize conditions for the collection of oral tissue specimens for the measurement of cAMP content.

The role of cAMP in oral tissue metabolism has been demonstrated by Grower, *et al.*,[3] in an *in vivo* study of gingival wounds in Rhesus monkeys. They reported a coordinate increase in collagen and cAMP content as healing progressed. Decreased concentrations of cAMP were found to be associated with proliferation of granulation tissue; only after cAMP concentrations increased did differentiation and tissue

maturation proceed. In addition, Schaeffer, *et al.*,[4] using biopsies of human gingiva, found that tissue from subjects with periodontitis had significantly lower cAMP levels than uninflamed tissue.

Bourne, *et al.*,[5] have proposed that cAMP acts *in vivo* as a mediator of the character and intensity of inflammation and immune responses by an inhibitory action on the inflammatory and immunologic functions of leukocytes. In addition, Ichikawa, *et al.*,[6] showed that exogenous cAMP was able to inhibit the formation of carrageenin induced granuloma and inflammation in rats.

The normal growth of the epithelial cells apparently is also controlled by the intracellular levels of cAMP. Voorhees, *et al.*,[7] reported that the hyperplastic and abnormally differentiated epidermis of psoriasis exhibited decreased levels of cAMP from that seen in normal skin. They proposed that the rate of epidermal proliferation is inversely proportional to intra-epidermal cAMP levels. In addition, cAMP apparently also regulates collagen synthesis by fibroblasts. Hsie, *et al.*,[8] observed that 1 mM dibutyryl cAMP produced a six to ten-fold increase in ^{3}(H) proline incorporation into collagen in CHO cells in tissue culture. Green, *et al.*,[9] have previously shown that virally transformed fibroblasts have low levels of cAMP and synthesize less collagen than normal cells. Peterkofsky and Prather[10] confirmed this observation in transformed cells and found that dibutyryl cAMP increased collagen synthesis in transformed cells. Goggins, *et al.*,[11] have reported similar results in transformed cells with respect to mucopolysaccharide synthesis as measured by (^{35}S) sulfate incorporation.

MATERIALS AND PROCEDURES

Twelve monkeys were used in this study to determine the following parameters:

1. The normal variation in basal gingival cAMP levels between the right and left quadrants of uninfiltrated tissues.

2. Effects that infiltration of the attached gingiva with Lidocaine, Lidocaine with 1:100,000 epinephrine, and saline caused on gingival cAMP levels.

3. The rapidity of onset and duration of changes in gingival cAMP levels caused by infiltration with Lidocaine containing 1:100,000 epinephrine.

The Rhesus monkeys used were pre-sedated with IM Sernlyn and anesthetized with IV sodium pentobarbital during all experimental procedures. A 1 to 2 ml sample of venous blood was then collected in heparinized tubes (prior to any experimental procedures) for measurement of basal plasma cAMP levels. All experiments were done in a paired design so that each animal served as its own control. At the start of any experiment (time 0) the facial attached gingiva on the left side of the maxilla or mandible (from the mesial of the canine to the distal of the second molar or from the mesial of the central incisor to the mesial of the canine) were surgically removed using intrasulcular incisions followed by periosteal elevation. The excised tissues were rinsed of excess blood, placed in plastic tissue containers, and frozen in liquid nitrogen. The attached gingiva on the corresponding areas of the right side of the maxilla or mandible were

then infiltrated with the agent being tested and surgically removed after the appropriate time period had elapsed (30 seconds to 10 minutes). These tissues were also rinsed of excess blood and frozen in liquid nitrogen.

After collection of all the tissue samples the wet weights of the frozen samples were determined, and the samples cooled to -120°C in liquid nitrogen. The frozen gingival samples were then pulverized in a steel mortar and pestle cooled to -40°C. (The pulverization was necessary in order to facilitate extraction of cAMP from the tissues due to the fibrous nature of the tissue.) After pulverization, the gingival samples were placed in Potter Elvehjem glass homogenizing tubes maintained in an ice bath at 4°C, and 2 ml of ice-cold 6% TCA (trichloroacetic acid) was added. In addition, 0.1 ml of H_2O containing 0.0022 μC of (^3H) cAMP (Adenosine 3', 5' cyclic phosphate [8 -^3H], SA 20 c/mMole, Schwarz/Mann) was added to monitor the recovery of cAMP through the extraction procedures. The cyclic nucleotides in the tissue were extracted by homogenizing the tissue with a motor driven teflon pestle. The blood samples which were collected were centrifuged at 4000 RPM at 4°C to separate the plasma from the red blood cells. The plasma was then removed, the recovery marker was added, and an equal volume of 10% TCA was added to the plasma to precipitate the proteins and extract the cAMP in the plasma. The homogenates and plasma samples were transferred to conical glass centrifuge tubes and centrifuged for 15 minutes at 4000 RPM at 4°C to separate the precipitated proteins from the cyclic nucleotides present in the 6% TCA extract.

The TCA extracts were extracted three times with 5 ml of ethyl ether to remove the TCA present. The resulting aqueous solutions were evaporated to dryness in a stream of air in a 60°C waterbath. The residues were redissolved in 1 ml of pH 6.2 acetate buffer and the samples were assayed for their cAMP content using the Schwarz/Mann[12] cyclic AMP radioimmunoassay kit (^{125}I) according to the procedures described by Steiner, *et al.*[13] The radioactivity in the (^{125}I) nucleotide antigen-antibody complexes obtained in the assays was counted in a Packard Auto-Gamma Spectrometer. The cAMP radioimmunoassay was sensitive enough to measure from 0.01 to 10 picomoles of cAMP per sample. The efficiency of the extraction procedures for the cAMP content of gingiva was found to average 75±2% (mean ± SE of 8 monkeys) based on the recovery of the (^{3}H) cAMP tracer added to the samples. In addition, the specificity of the radioimmunoassay for cAMP was determined by fractionating the extracted nucleotides on 1.0 ml column of BioRad AG-1-X8, 200-400 mesh ion exchange resin equilibrated in 0.1N formic acid. The columns were washed with 10 ml of 0.1N formic acid and the cAMP was eluted with 10 ml of 2N formic acid. The elute was lyophilized and the residue redissolved in 1 ml of acetate assay buffer for analysis of the cAMP content. It was found that 79±3% (mean ± SE of eight observations) of the total cAMP content of the original gingival extract (as determined by radioimmunoassay) was eluted in the purified cAMP fraction.

The precipitated proteins remaining after TCA extraction of the gingiva and plasma were resuspended in 2 ml of 1.0N NaOH, incubated

at 60°C for two hours to solubilize them, and the protein content of the samples was determined by the micro method of Lowry, *et al.*[14]

Statistical significance was analyzed by the students' t-test for paired data using a two-tailed test.[15]

RESULTS

The average cAMP content of the uninjected control monkey gingiva and blood plasma are shown in Table I. Table I shows that the cAMP content of monkey gingiva is 75 times greater than that seen in the blood plasma.

Measurement of the basal levels of cAMP in the uninjected gingiva of the right and left sides of the mandibles of four monkeys did not reveal any significant differences as seen in Figure 1. Infiltration of the attached gingiva with saline for five minutes caused only a 25% increase in gingival cAMP levels and infiltration with plain Lidocaine caused a 20% increase in cAMP levels versus the control tissues as seen in Figure 1. None of these increases were statistically significant.

The only significant increase in gingival cAMP levels was caused by infiltration with 2% Lidocaine containing 1:100,000 epinephrine. Figure 1 shows that the cAMP levels of tissues infiltrated for 5 min. with Lidocaine containing epinephrine were 1100% higher than the uninjected control tissues ($p < 0.005$, $n=4$). The cAMP levels of blood plasma samples taken 5 min. after infiltration did not show any differences from the control levels seen in Table I.

Figure 2 summarizes the rate, magnitude, and duration of the effect that infiltration of the attached gingiva with 2% Lidocaine containing 1:100,000 epinephrine had on gingival cAMP levels. The results are expressed both on the basis of picomoles of cAMP/mg wet wt of gingiva (diagonal lines) and on the basis of picomoles of cAMP/mg of protein

(horizontal lines). Figure 2 shows that within 30 seconds of infiltration a 250% increase in gingival cAMP levels was detected. The levels reached their maximum 5 min. after injection, at which time the levels were 1000-1100% greater than the control levels. At 10 min. the levels were lower than the peak level seen at 5 min., but were still 500-600% greater than the basal levels of cAMP.

DISCUSSION

The results expressed in Figure 1 show that the increase in tissue cAMP levels after infiltration anesthesia was due to the epinephrine contained in the anesthesia. Neither plain Lidocaine nor saline had a significant effect. The small effect that was seen with saline or plain Lidocaine may have been due to tissue ischemia which can cause localized production of epinephrine, which can result in cAMP production.[16]

The concentration of epinephrine in the Lidocaine used in this experiment was calculated to be 5.5×10^{-8}M which, as can be seen in Figure 2, produced a 10 to 11-fold increase over basal cAMP levels. These results were similar to those of Yoshikawa, *et al.*[1] who found that incubating pig or human epidermal slices *in vitro* (dermis + epidermis) with 5×10^{-5}M epinephrine caused a 20-fold increase in tissue cAMP levels. The increase in cAMP levels was caused by the activation of the enzyme adenyl cyclase which converts tissue ATP to cAMP.[17] They observed stimulation of cAMP production with concentration of epinephrine between 10^{-8}M to 10^{-4}M with the maximal activation of cAMP production at 5×10^{-6}M epinephrine.

The levels of cAMP determined in this *in vivo* study were from tissues composed of a mixed population of cells. Therefore, the question of which cells were the source of the tissue cAMP, and which cell types

showed the most changes, has to be considered. Voorhees, *et al.*[7] found the levels of cAMP in human epidermis to be 12.3 ± 1.7 picomoles of cAMP/mg protein, while that of whole blood was 300 times lower, i.e., 0.042 ± .006 picomoles of cAMP/mg protein.[18] Sheppard[19] found that the content of cAMP in fibroblasts (3T3) in culture was 24.4 ± 3.4 picomoles cAMP/mg protein. The levels of cAMP determined in monkey gingiva in this study ranged from 12-20 picomoles cAMP/mg of protein and were thus similar to that seen in skin and fibroblasts, but 75-fold higher than seen in blood plasma (Table I). Thus, the tissue levels measured, and their increases due to epinephrine, were of epithelial and collagenous origin and not due merely to changes in blood volume of the tissues or increases in plasma cAMP levels. In addition, Ball, *et al.*[20] observed that infusion of epinephrine IV into human volunteers caused only a 2 to 4-fold increase in plasma cAMP levels, which is far less than the increased levels of cAMP observed in infiltrated gingiva (Figures 1, 2).

A question of considerable importance is what are the consequences and therapeutic applications of the increase seen in gingival cAMP levels (Figure 2) after infiltration anesthesia with Lidocaine containing epinephrine. Since the repair of oral tissues after wounding as well as repair following periodontal surgery includes both changes in the collagen and epithelial components of the tissues,[21] the effects of cAMP on these tissues will be considered.

In vitro experiments utilizing fibroblast cultures have shown that intracellular concentrations of cAMP regulate growth rate,[22-24] contact inhibition,[25] morphology,[24,26,27] and adhesiveness to substratum.[24] Cyclic AMP apparently also regulates collagen synthesis by

fibroblasts.[8]

The initial repair of gingival flaps after periodontal surgery occurs through collagen fiber production by pre-existing fibroblasts.[28] The increase seen in gingival cAMP levels after injection of epinephrine containing anesthetics may serve to stimulate this process, since Hsie, *et al.*[8] demonstrated that addition of 1 mM dibutyryl cAMP to cultures of CHO cells caused a six to ten-fold increase in (^{3}H) proline incorporation into collagen in these cells.

The normal growth of epithelial cells apparently is also controlled by the levels of cAMP found in them. Voorhees, *et al.*[7] reported that hyperplastic and abnormally differentiated epidermis of psoriasis exhibited decreased levels of cAMP from that seen in normal skin. They proposed that the rate of epidermal proliferation may be inversely proportional to intra-epidermal cAMP levels. The lowered gingival cAMP levels reported by Grower, *et al.*[29] and Schaeffer, *et al.*[4] in inflamed gingival tissue may be one of the factors causing the overgrowth of epithelium into periodontal pockets. The increased levels of cAMP occurring after infiltration with epinephrine (Figure 2) may cause a slight inhibition to the downgrowth of epithelium into the periodontal pocket after surgery. This possible action of cAMP on crevicular epithelium also suggests a model for the development of treatment modalities to retard or prevent the growth of epithelium into periodontal pockets.

Cyclic AMP also appears to play a role in the modulation of inflammation and immunity. Bourne, *et al.*[5] have proposed that *in vivo* mediators of inflammation may regulate the character and intensity of inflammatory and immune responses by a general inhibitory action of

cAMP on the inflammatory and immunologic functions of leukocytes. In addition, *in vivo* Ichikawa *et al.*,[6] showed that exogenous cAMP was able to inhibit the formation of carrageenin induced granuloma and inflammation in the rats. The local increase seen in tissue cAMP levels after infiltration with epinephrine (Figure 2) may act to reduce the inflammatory response of the tissues by preventing an increased influx of inflammatory cells into the tissues. This hypothesis is supported by Hill, *et al.*[30] who reported that increased intracellular concentrations of cAMP inhibited the leukotactic response of neutrophils to bacterial chemotactic factor. The increased levels of cAMP in the tissues might also prevent the selective secretion of lysosomal enzymes by the polymorphonuclear leukocytes, a response which can be inhibited by agents which increase intracellular levels of cAMP.[31]

The results of this study point out that in the execution of clinical studies in which the levels of cAMP are measured, care must be taken not to use any anesthetics containing epinephrine. The use of epinephrine containing anesthetics will result in markedly elevated tissue levels of cAMP (Figures 1,2) which would result in the drawing of erroneous inference. In addition, the tissues that are collected should be immediately frozen in liquid nitrogen or dry ice, since Yoshikawa, *et al.*[16] reported that maintaining excised tissues at room temperature resulted in a rapid increase in tissue cAMP levels which was most pronounced 2 min after removal.

While it is proposed that increasing the tissue levels of cAMP

in vivo may serve to increase collagen production and reduce excess epithelial cell proliferation and inflammation, this hypothesis is based partly on circumstantial evidence. Considerably more work needs to be done to validate this hypothesis.

SUMMARY

The basal levels of cAMP in the attached gingiva of Rhesus monkeys and the changes in tissue cAMP levels produced by infiltration anesthesia with Lidocaine and Lidocaine containing 1:100,000 epinephrine was studied. The basal level of cAMP in uninjected monkey gingiva ranged from 12-20 picomoles cAMP/mg of gingival protein. This level was 75 times greater than the cAMP content of monkey blood plasma.

Infiltration of the attached gingiva with saline or plain Lidocaine for 5 min. did not produce any significant changes in tissue cAMP levels. Infiltration of the gingiva with Lidocaine containing 1:100,000 epinephrine, on the other hand, caused a very marked increase in tissue cAMP levels. Thirty seconds after infiltration with Lidocaine with 1:100,000 epinephrine there was a 150% increase in cAMP content of the anesthetized tissue versus the uninjected control tissues. The maximal increase in tissue cAMP levels was observed 5 min. after infiltration when the cAMP content of the gingiva was 1000-1100% above the control level.

It is proposed that regulation of tissue cAMP levels by epinephrine or other agents may prove of therapeutic usefulness in regulating inflammation and healing of tissues after surgery or other trauma.

In conducting research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council.

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TABLE I

cAMP CONTENT OF MONKEY GINGIVA AND PLASMA

SAMPLE	Picomoles cAMP / mg Protein
Gingiva	18.81 ± 1.70[a]
Plasma	0.24 ± 0.09[b]

a - Mean ± SE of 17 observations from the maxillary gingiva of 8 monkeys.

b - Mean ± SE of 6 monkeys.

FIGURE 1. Effects of Different Treatments on Gingival cAMP Levels.

The cAMP content of the gingival tissues was determined as the picomoles of cAMP/mg of gingival protein and then expressed as the percent of the control level. In the Figure, the control level of cAMP is expressed as 100. The specific treatments used in each group were as follows:

r vs. l - The attached facial gingiva of the right and left sides of the mandible (mesial of canine to distal of second molar) of four monkeys was removed without any treatment. The gingiva on the left side was used as the control side in expressing the results shown.

Sal - The attached facial gingiva of the right side of the maxilla of four monkeys (mesial of right central to mesial of canine) was injected with 0.5 cc saline 5 min before collection of the tissue, while the same area of the left side of the maxilla was the uninjected control.

Lido - The attached facial gingiva of the right side of the maxilla of four monkeys (distal of the canine to distal of the second molar) was injected with 0.5 cc of plain Lidocaine 5 min before collection of the tissues while the same area of the left side of the maxilla served as the uninjected control.

Lido + epi - The attached facial gingiva of the right side of the maxilla of four monkeys (distal of canine to distal of second molar) was infiltrated with 0.5 cc of 2% Lidocaine containing 1:100,000 epinephrine 5 min before collection of the tissues while the same area of the left side of the maxilla served as the uninjected control.

Figure 2. Onset of Action and Duration of the Effect that Infiltration of Lidocaine with Epinephrine has on Gingival cAMP Levels.

The changes in cAMP content of the gingival samples are expressed as the percent of the control level. The control level of cAMP was taken as 100. The results are expressed based on both the picomoles cAMP/mg of protein in the sample (horizontal bars) and on the picomoles cAMP/mg of wet wt of gingiva (diagonal lines).

At time 0 the facial attached gingiva of the right side of the mandible of four monkeys (from the mesial of the canine to the distal of the second molar) was infiltrated with 0.5cc of 2% lidocaine containing epinephrine. The gingiva on the left side served as the uninjected control and was removed immediately after infiltration of the right side. Thirty seconds after injection of the right side, the gingiva from the mesial of the canine to the mesial of the first bicuspid, was surgically removed, rinsed, and frozen in liquid nitrogen. Sixty seconds after injection, the gingiva from the mesial of the first bicuspid to the distal of the second bicuspid was removed and 120 seconds after injection the gingiva from the mesial of the first molar to the distal of the second molar was removed and frozen. The attached gingiva on the right side of the maxilla and mandible of four additional monkeys (distal of canine to distal of second molar) was infiltrated with 0.5cc of lidocaine containing 1:100,000 epinephrine. The maxillary gingiva was excised five minutes after infiltration, while the mandibular gingiva was excised ten minutes after infiltration. The uninjected gingiva of the left side of the maxilla and mandible served as the control samples.

Figure1

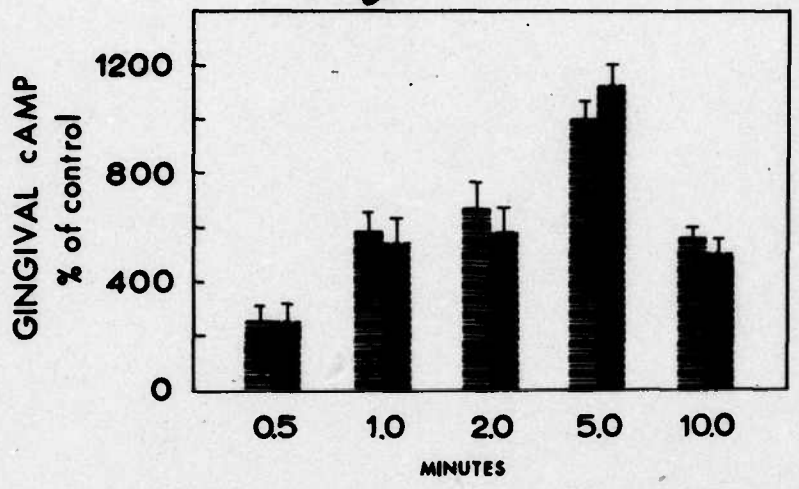
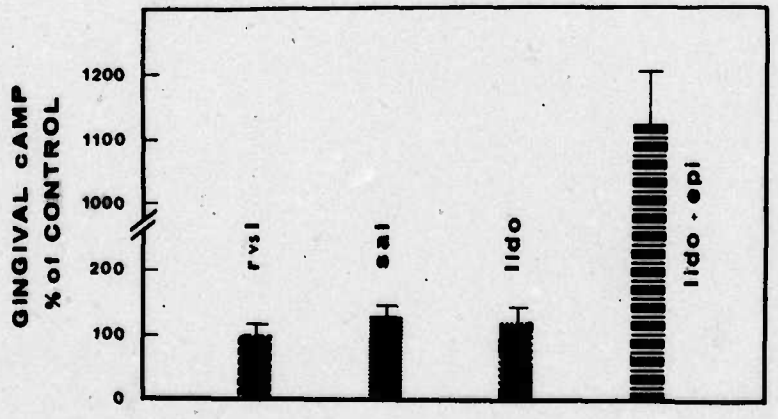
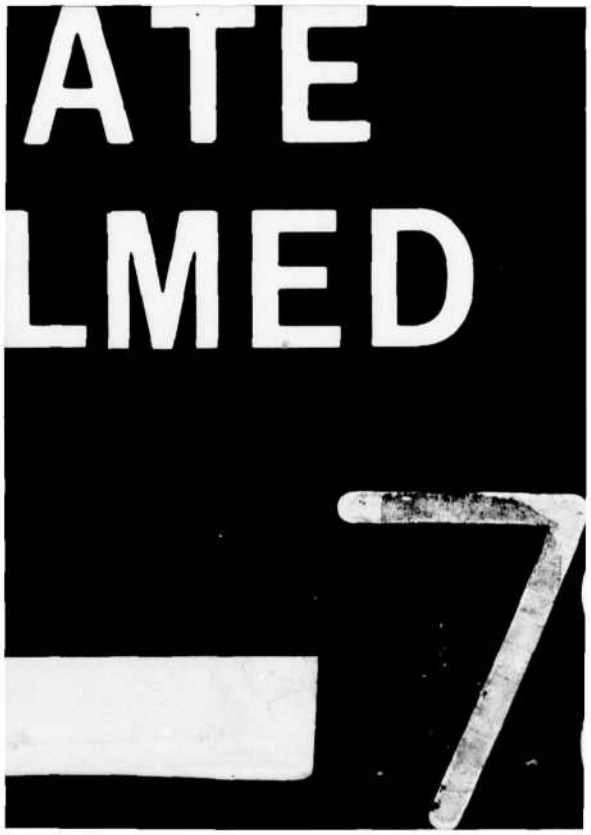


Figure2

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